# INTRAMYOCARDIAL INJECTION OF AUTOLOGOUS BONE MARROW


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Abstract of **WO03101201**

Methods are provided for enhancing transfection efficiency of bone marrow cells by transfecting early attaching cells derived from bone marrow in culture. Methods are also provided for utilizing such early attaching cells derived from autologous bone marrow to deliver angiogenesis-promoting transgenes to a patient. The transfected early attaching cells are introduced into an ischemic tissue, such as the heart, to enhance formation of collateral blood vessels. Methods are also provided for treating ischemic muscle by co-administration of autologous bone marrow cells and RGTA, for example RGTA11.

# INTRAMYOCARDIAL INJECTION OF AUTOLOGOUS BONE MARROW

Description of **WO03101201**

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INTRAMYOCARDIAL INJECTION OF AUTOLOGOUS BONE MARROW
FIELD OF THE INVENTION[0001] This application is directed to methods for injecting autologous bone marrow and bone marrow cells. More specifically, this invention is directed to intramyocardial injection of autologous bone marrow and transfected bone marrow cells to enhance collateral blood vessel formation and tissue perfusion.

BACKGROUND OF THE INVENTION[0002] The use of recombinant genes or growth factors to enhance myocardial collateral blood vessel function may represent a new approach to the treatment of cardiovascular disease.Komowski, R. , et al. ,"Delivery strategies for therapeutic myocardial angiogenesis,"Circulation 2000; 101: 454-458. Proof of concept has been demonstrated in animal models of myocardial ischemia, and clinical trials are underway. Unger, E. F., et al. ,"Basic fibroblast growth factor enhances myocardial collateral flow in a caninemodel,"Am JPhysiol 1994; 266:H1588-1595 ; Banai, S. etal.,"Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs,"Circulation 1994; 83-2189 ; Lazarous, D. F. , et al. ,"Effect of chronic systemic administration of basic fibroblast growth factor on collateral development in the canine heart,"Circulation 1995; 91: 145-153; Lazarous, D. F. , et al., "Comparative effects of basic development and the arterial response to injury," Circulation 1996; 94:1074-1082 ; Giordano, F. J. , et al.,"Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart,"Nature Med 1996; 2: 534-9. Most strategies for trans-catheter delivery of angiogenic factors have employed an intracoronary route that may have limitations due to imprecise localization of genes or proteins and systemic delivery to non-cardiac tissue. Thus, it would be desirable to have the capacity for direct delivery of angiogenic factors or genes to precisely defined regions of the myocardium rather than to the entire heart, and to minimize the potential for systemic exposure. Guzman, R. J. , et al. , "Efficient gene transfer into myocardium by direct injection of adenovirus vectors, " Circ Res 1993; 73: 1202-7; Mack, C. A. , et al. ,"Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for

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VEGF-121, improves myocardial perfusion and function in the ischemic porcine heart,"J Thorac Cardiovasc Surg 1998; 115: 168-77.

[0003] The effect of direct intra-operative intramyocardial injection of angiogenic factors on collateral function has been studied in animal models of myocardial ischemia. Open chest, transepicardial administration of an adenoviral vector containing a transgene encoding an angiogenic peptide resulted in enhanced collateral function. (Mack et al., supra.) Angiogenesis was also reported to occur with direct intramyocardial injection of an angiogenic peptide or a plasmid vector during open heart surgery in patients.

Schumacher, B. , et al.,"Induction of neoangiogenesis in ischemic myocardium by human growth factors. First clinical results of a new treatment of coronary heart disease," Circulation1998 ; 97: 645-650; Losordo, D. W., et al. , "Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia,"Circulation 1998; 98:2800.

[0004] Despite the promising hope for therapeutic angiogenesis as a new modality to treat patients with coronary artery disease, there is still a huge gap regarding what specific strategy will optimally promote a clinically relevant therapeutic angiogenic response.

Moreover, it is unclear which one (or more) out of multiple angiogenic growth factors may be associated with a beneficial angiogenic response. In addition, the use of different tissue delivery platforms, e. g. , proteins, adenovirus,or"naked"DNA, to promote the optimal angiogenic response has remained an open

issue.

SUMMARY OF THE INVENTION[0005] Most currently tested therapeutic approaches have focused on a single angiogenic growth factor (e. g. , VEGF, FGF,angiopoietin-1) delivered to the ischemic tissue. This can be accomplished either by delivery of the end-product (e. g. , protein) or by gene transfer, using diverse vectors. However, it is believed that complex interactions among several growth factor systems are probably necessary for the initiation and maintenance of new blood vessel formation. More specifically, it is believed important to induce a specific localized angiogenic milieu with various angiogenic cytokines interacting in

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concert and in a time-appropriate manner to initiate and maintain the formation and function of new blood vessels.

[0006] The bone marrow (BM) is a natural source of a broad spectrum of cytokines (e. g., growth factors) and cells that are involved in the control of angiogenic processes. It is therefore believed that the intramyocardial injection of autologous (A) BM or bone marrow cells derived therefrom, by taking advantage of the natural ability of these cells to secrete many angiogenic factors in a time-appropriate manner, provides an optimal intervention for achieving therapeutic collateral development in ischemic myocardium.

[0007] According to various embodiments of the invention, autologous bone marrow, or cells derived therefrom, is injected, either as a"stand alone"therapeutic agent or combined with any pharmacologic drug, protein or gene or any other compound or intervention that may enhance bone marrow production of angiogenic growth factors and/or promote endothelial cell proliferation, migration, and blood vessel tube formation.

The"combined"angiogenic agents can be administered directly into the patient or target tissue, or incubated ex-vivo with bone marrow prior to injection of bone marrow into the patient. Non-limiting examples ofthese"combined"angiogenic agents are Granulocyte- Monocyte Colony Stimulator-y Factor (GM-CSF), Monocyte Chemoattractant Protein 1 (MCP-1), and Hypoxia Inducible Factor-1 (HIF-1).

[0008] In one embodiment according to the invention, filtered bone marrow and/or bone marrow cells (either transfected with a gene encoding an angiogenic factor, or not) is directly administered into the ischemic target tissue accompanied by an angiogenic agent known as RGTA (ReGeneraTing Agent). RGTA is a family of agents that has properties mimicking thoseof heparan sulfates toward heparin-binding growth factors (or HBGF) and which stimulate tissue repair and protection. For example, one member of the RGTA family,RGTA 11, has previously been shown to prevent most of the damage resulting from acute skeletal muscle ischemia (FASEB J. (1999) 13: 761-766). In addition, it has been shown that injectionof RGTAI I into ischemic heart tissue in a pig infarction model caused increase in the number of blood vessels therein, as well as recovery of84% of the initial left ventricular ejection fraction, and an almost 50% reduction in the infarct size.

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[0009] RGTA11, a chemically defined member of the RGTA family, is a Dextran derivative obtained by controlled and sequential substitutions on the glucose residues of Dextran T as previously described (M. Mauzac and J. Josefonvicz (1984) Biomaterials 5: 301-304). In RGTA11, the percentages of substitutions are 69,2. 5 and 36.5% for the carboxymethyl, carboxymethylbenzylamine, and carboxymethyl-benzylaminde sulfonate and sulfate groups, respectively. The anticoagulant activity ofRGTA 11 is 3.5 IU/mg (compared with heparin, usually at about170-180IU/mg). Although simultaneous injection is not required in practice of the invention methods,RGTA11 can conveniently be injected simultaneously with autologous bone marrow cells, either fresh or treated as described herein, into a site of muscle ischemia to promote growth of blood vessels therein.

[0010] For example,RGTA11 can be added to a carrier solution containing filtered bone marrow cells prior to injection of the cells. If theRGTAI I is added to the carrier containing the bone marrow, since it has activity as a blood anticoagulant, the amount of heparin administered with the bone marrow cells, if

present, can be reduced accordingly.

[0011] Another example of an intervention that enhances bone marrow production of angiogenic factors is ex-vivo exposure of bone marrow cells to hypoxia. The autologous bone marrow, alone or with"combined"angiogenic agents, can be delivered to the patient directly via either trans-endocardial or trans-epicardial approaches into either ischemic and/or non-ischemic myocardium, or directly into any other ischemic organ (including a peripheral limb) to enhance and/or promote the development of collateral blood vessel formation and therefore collateral flow to ischemic myocardium or ischemic limbs. This approach can also be used to promote the development of newly implanted dedifferentiated and/or differentiated myocardial cells by the process of cardiac myogenesis.

[0012] Accordingly, in one embodiment, the invention provides methods for enhancing transfection efficiency in bone marrow cells. In this embodiment, bone marrow cells are cultured under suitable culture conditions in a container for a period of time sufficient to promote production by the bone marrow of early attaching cells. The

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early attaching cells are transfected with a vector comprising a polynucleotide that encodes one or more angiogenic cytokines, growth factors or mammalian angiogenesis- promoting factors. In the invention method, the efficiency with which the early attaching cells are transfected is enhanced as compared with that obtained by transfecting fresh bone marrow or non-adherent bone marrow cells with the vector.

[0013] In another embodiment, the invention provides methods for enhancing collateral blood vessel formation in a subject in need thereof by obtaining autologous bone marrow from the patient, growing the autologous bone marrow under suitable culture conditions in a container for a period of time sufficient to promote production by the bone marrow of early attaching cells, and transfecting the early attaching cells with a vector comprising a polynucleotide that encodes one or more angiogenic cytokines, growth factors or mammalian angiogenesis-promoting factors. An effective amount of the transfected cells is directly administered to a desired site in the patient to cause expression of the one or more agents leading to enhanced collateral blood vessel formation at the site in the patient.

[0014] In yet another embodiment, the invention provides compositions comprising early attaching cells derived from autologous bone marrow that have been transfected with a vector comprising a polynucleotide that encodes one or more cytokines, growth factors or mammalian angiogenesis-promoting factors as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS[0015] Fig.1 is a graph of the proliferation of PAEC's vs. the quantities of conditioned medium;[0016] Fig. 2 is a graph of the proliferation of endothelial cells vs. the quantities of conditioned medium;[0017] Fig. 3 is a graph of the concentration of VEGF in conditioned medium over a four-week period of time; and[0018] Fig. 4 is a graph of the concentration of MCP-1 in conditioned medium over a four-week period of time.

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DETAILED DESCRIPTION OF THE INVENTION[0019] Bone marrow is a natural source of a broad spectrum of cytokines, growth factors and angiogenesis-promoting factors that are involved in the control of angiogenic and inflammatory processes. The angiogenic cytokines, growth factors and angiogenesis-promoting factors expressed comprise mediators known to be involved in the maintenance of early and late hematopoiesis(IL-lalpha and IL-lbeta,IL-6,IL-7,IL-8, IL- 11 and IL-13; colony-stimulating factors, thrombopoietin, erythropoietin, stem cell factor, fit 3-ligand, hepatocyte cell growth factor, tumor necrosis factor alpha, leukemia inhibitory factor, transforming growth factors beta 1 and beta 3; and macrophage inflammatory protein 1 alpha), angiogenic factors (fibroblast growth factors 1 and 2, vascular endothelial growth factor) and mediators whose usual target (and source) is the connective tissue-forming cells (platelet-derived growth factor A, epidermal growth factor, transforming growth factors alpha and beta 2, oncostatin M and insulin-like growthfactor-1), or neuronal cells (nerve growth factor). Sensebe, L. , etal., Stem Cells 1997; 15: 133-43. Moreover, it has been shown that VEGF polypeptides are present in platelets andmegacaryocytes, and are released from activated platelets together with the release of beta-thromboglobulin. Wartiovaara, U. , et al.,Thromb Haemost 1998 ; 80: 171- 5; Mohle, R.,Proc Natl Acad Sci

USA 1997 ; 94: 663-8.

[0020] There are also indicators to support the concept that angiogenesis is needed to support bone marrow function and development of hematopoietic cells, including stem cells and progenitor cells, that may enter the circulation and target to sites of wound healing and/or ischemia, ultimately contributing to new blood vessel formation.

Monoclonal antibodies that specifically recognize undifferentiated mesenchymal progenitor cells isolated from adult human bone marrow have been shown to recognize cell surface markers of developing microvasculature, and evidence suggests such cells may play a role in embryonal angiogenesis. Fleming, J. E. , Jr. , Dev Dyn1998 ; 212: 119- 32.

[0021] Bone marrow angiogenesis may become exaggerated in pathologic states where the bone marrow is being activated by malignant cells (such as in multiple myeloma) where bone marrow angiogenesis has been shown to increase simultaneously

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with progression of human multiple myeloma cells. Ribatti, D. , et al., Br J Cancer 1999; 79: 451-5. Moreover, vascular endothelial growth factor (VEGF) has been shown to play a role in the growth of hematopoietic neoplasms such as multiple myeloma, through either a paracrine or an autocrine mechanism. Bellamy, W. T., CancerRes 1999 ; 59: 728- 33; Fiedler, W., Blood 1997; 89: 1870-5). It is believed that autologous bone marrow, with its unique native humoral and cellular properties, is a potential source of various angiogenic compounds. This natural sourceof"mixed"angiogenic cytokines can surprisingly be utilized as a mixture of potent interactive growth factors to produce therapeutic angiogenesis and/or myogenesis; use of the cells per se could provide a more sustained source of these natural angiogenic agents.

[0022] One of the angiogenesis-promoting factors that most likely participate in initiating angiogenesis in response to ischemia is HIF-1, a potent transcription factor that binds to and stimulates the promoter of several genes involved in responses to hypoxia.

Induction and activation ofHIF-I is tightly controlled by tissuepO2 ;HIF-1 expression increases exponentially aspO2 decreases, thereby providing a positive feedback loop by which a decrease inPO2 causes an increase in the expression of gene products that serve as an adaptive response to a low oxygen environment. Activation of HIF-1 leads, for example, to the induction of erythropoietin, genes involved in glycolysis, and to the expression of VEGF. It probably also modulates the expression of many other genes that participate in the adaptive response to lowp02 levels. The mechanism by whichHIF-1 regulates levels of proteins involved in the response to hypoxia is through transcriptional regulation of genes responding to lowPO2. Thus, such genes have short DNA sequences within the promoter or enhancer regions that contain HIF-1 binding sites, designated as hypoxia responsive elements (HRE). HIF-1 is a heterodimer with a basic helix-loop-helix motif, consisting of the subunits HIF-la andHIF-1 (3. Its levels are regulated byp02 both transcriptionally andposttranscriptionally-HIF-1 induction is increased by hypoxia, and its half-life is markedly reduced as pO2 levels increase.

[0023] It is relevant that while expression of HIF-1 (as determined in HeLa cells) is exponentially and inversely related top02, the inflection point of the curve occurs at an oxygen saturation of5%, with maximal activity at 0.5%and ! 4 maximal activity at 1.5-

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2.0%. These are relatively low levels of hypoxia, and it is not clear whether such levels occur in the presence of mild levels of myocardial or lower limb ischemia-i. e. , levels present in the absence of tissue necrosis (myocardial infarction, and leg ulcerations, respectively). Thus, bone marrow cells could have the capacity to secrete angiogenic factors and thereby enhance collateral development. However, it is possible that such activity may not become manifest in the specific tissue environments treated unless some additional stimulus is present. It is, therefore, a preferred aspect of the invention to co administer, if necessary, bone marrow implant with HIF-1. It is anticipated that HIF-1 will provide optimal expression of many of the hypoxia-inducible angiogenic genes present in the bone marrow implant. The HIF-1 can be injected either as the protein, or as the gene. If as the latter, it can be injected either in a plasmid or viral vector, or any other manner that leads to functionally relevant protein levels. It is emphasized, however,

thatHIF-1 is used as an example of an intervention that could enhance production of angiogenic substances by bone marrow. This invention also covers use of other angiogenic agents, which by enhancingHIF-1 activity (i. e. , prolonging its half-life), or by producing effects analogous to HIF-1, stimulate the bone marrow to increase expression of angiogenic factors. A similar approach involves the exposure of autologous bone marrow to endothelial PAS domain protein 1 (EPAS 1). EPAS 1 shares high structural and functional homology withHIF-1 and is also known as HIF-2.

[0024] In another embodiment according to the invention, to enhance VEGF promoter activity, by HIF-1, bone marrow cells can be exposed ex-vivo in culture to hypoxia or other forms of energy, such as, for example, ultrasound, RF, or electromagnetic energy.

This intervention increases VEGF and other gene expression. By this effect it may augment the capacity of bone marrow to stimulate angiogenesis. Thus, in this embodiment, the invention involves the ex-vivo stimulation of aspirated autologous bone marrow byHIF-1 (or products that augment the effects of HIF-1 or produce similar effects to HIF-1 on bone marrow) or direct exposure of bone marrow to hypoxic environment followed by the delivery of activated bone marrow cells to the ischemic myocardium or peripheral organ (e. g. , ischemic limb) to enhance collateral-dependent perfusion in cardiac and/or peripheral ischemic tissue.

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[0025] Current data indicate the importance of monocyte-derived cytokines for enhancing collateral function. Monocytes are activated during collateral growth in vivo, and monocyte chemotactic protein-1 (MCP-1) is upregulated by shear stress in vitro. It has been shown that monocytes adhere to the vascular wall during collateral vessel growth (arteriogenesis) and capillary sprouting (angiogenesis). MCP-1 was also shown to enhance collateral growth after femoral artery occlusion in the rabbit chronic hindlimb ischemia model (Ito et al., Circ Res 1997; 80: 829-3). Activation of monocytes seems to play an important role in collateral growth as well as in capillary sprouting. Increased monocyte recruitment by LPS is associated with increased capillary density as well as enhanced collateral and peripheral conductance at 7 days after experimental arterial occlusion (Arms M. et al.,J Clin Invest 1998 ; 101 : 40-50.).

[0026] A further aspect of the invention involves the ex-vivo stimulation of aspirated autologous bone marrow by MCP-1, followed by the direct delivery of activated bone marrow cells to the ischemic myocardium or peripheral organ (e. g. , ischemic limb) to enhance collateral-dependent perfusion and muscular function in cardiac and/or peripheral ischemic tissue. The stimulation of the bone marrow could be by the direct exposure of the bone narrow to MCP-1 in the form of the protein, or the bone marrow cells can be transfected with a vector carrying the MCP-1 gene. For example, bone marrow, or early attaching cells derived from bone marrow, can be transfected with a plasmid vector, or with an adenoviral vector, carrying the MCP-1 transgene.

[0027] Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Granulocyte-Colony Stimulatory Factor (G-CSF) are stimulatory cytokines for monocyte maturation and are multipotent hematopoietic growth factors, which are utilized in clinical practice for various hematological pathologies such as depressed white blood cell count (i. e. , leukopenia or granulocytopenia or monocytopenia) which occurs usually in response to immunosuppressive or chemotherapy treatment in cancer patients. GM-CSF has also been described as a multilineage growth factor that induces in vitro colony formation from erythroid burst-forming units, eosinophil colony-forming units (CSF), and multipotential (CSF), as well as from granulocyte-macrophage CSF and granulocyte
CFU. (Bot F. J., Exp Hemato 1989,17 : 292-5). Ex-vivo exposure to GM-CSF has been

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shown to induce rapid proliferation of CD-34+ progenitor cells. (Egeland T. etal., Blood 1991; 78: 3192-g. ) These cells have the potential to differentiate into vascular endothelial cells and may naturally be involved in postnatal angiogenesis. In addition, GM-CSF carries multiple stimulatory effects onmacrophage/monocyte proliferation, differentiation, motility and survival (reduced apoptotic rate). Consistent with the combined known effects on bone marrow derived endothelial progenitor cells and monocytes, it is another aspect of the invention to use GM-CSF as an adjunctive treatment to autologous bone marrow injections aimed to induce new blood vessel formation and differentiation in ischemic

cardiovascular organs. Moreover, GM-CSF may further enhance therapeutic myocardial angiogenesis caused by bone marrow, by augmenting the effect of bone marrow, or by further stimulating, administered either in vivo or in vitro, bone marrow that is also being stimulated by agents such as HIF-1, EPAS 1, hypoxia, orMCP-1.

[0028] For example, ABM cells collected from a subject can be transfected, ex vivo, with a plasmid vector, or with an adenoviral vector, carrying an angiogenic cytokine growth factor or mammalian angiogenesis promoting factor transgene, such as the HIF-1 or EPSA1 transgene, for expression thereof in the cells and/or in the subject when the transfected cells are injected into a treatment site as described herein.

[0029] However, fresh bone marrow or bone marrow cells in solution can be difficult to transfect with a vector encoding the therapeutic cytokines, growth factors and angiogenesis-promoting factors described herein. To overcome this difficulty, it has been discovered that the efficiency with which bone marrow cells can be transfected ex vivo (e. g. in culture) with a vector carrying a transgene is greatly enhanced when bone marrow cells are grown in a container for a sufficient period of time to allow adherence of early attaching cells from the bone marrow to the container and the early attaching cells are selected for the transfection. "Early attaching cells"as the term is used herein means the cells from the culture medium containing bone marrow, or from bone marrow cells seeded into the container, that do not wash away after growth at suitable culture conditions for about 8 hours (e. g. , overnight) to about 24 hours. The early attaching cells are mostly monocytes, endothelial precursor cells, or other hematopoietic lineage cells.

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Inoculation takes place after culture of the cells for a period of from about 3 to about 28 days, for example after about 3 to about 14 days, or after about 3 to about 7 days. The early attaching cells can be inoculated with a vector encoding one or more angiogenic cytokines, growth factors and/or factors that promote angiogenesis in mammalian cell by any method known in the art, for example by in vitro contact for a period of about 2 hours to about 3 days after the inoculation. The vector used can be selected from any of those known in the art and include, but without limitation thereto, those described herein. The vector (e. g. a virus) is generally washed out about 2 hours to about 3 days after the inoculation before the cells are prepared for administration to the patient.

[0030] Optionally, the ABM can be filtered prior to placement in the container to remove particles larger than about 300g to about 20011. Bone marrow cells can also be separated from the filtered ABM for growth in the container leading to production of early attaching cells. Suitable culture conditions are well known in the art and include, but are not limited to, those described in the Examples herein.

[0031] Suitable transgenes for transfecting bone marrow-derived early attaching cells according to the invention methods include, but without limitation thereto, those encoding such angiogenesis-promoting agents as HIF-1, EPAS1 (also known as HIF-2), MCP-1, CM-CSF, and the like. An effective amount of the transfected early attaching cells derived from bone marrow prepared as described herein can be directly administered to (i. e. injected into) a desired site in a patient to enhance collateral blood vessel formation at the site in the patient. Particularly effective sites for administration of cells transfected with an angiogenesis-promoting agent include heart muscle or skeletal muscle, such as in the leg, to enhance collateral-dependent perfusion in cardiac and/or peripheral ischemic tissue.

[0032] In non-limiting illustration of the invention methods for obtaining enhanced transfection efficiency of bone marrow cells, studies have been conducted utilizing the X-gal transgene in an adenovirus vector to transfect bone marrow-derived early attaching cells prepared as described above. In these studies, staining of transfected cells with X-gal a suitable period of time after transfection shows that, compared with non-adherent

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bone marrow cells or fresh bone marrow, susceptibility of bone marrow-derived early attaching cells to transfection is substantially increased.

[0033] The polynucleotide encoding the therapeutic protein may be"functionally appended to", or"operatively associated with", a signal sequence that can"transport"the encoded product across the cell

membrane. A variety of such signal sequences are known and can be used by those skilled in the art without undue experimentation.

[0034] Gene transfer vectors (also referred to as"expression vectors") contemplated for such purposes are recombinant nucleic acid molecules that are used to transport nucleic acid into host cells for expression and/or replication thereof. Expression vectors may be either circular or linear, and are capable of incorporating a variety of nucleic acid constructs therein. Expression vectors typically come in the form of a plasmid that, upon introduction into an appropriate host cell, results in expression of the inserted nucleic acid.

[0035] Suitable viral vectors for use in gene therapy have been developed for use in particular host systems, particularly mammalian systems and include, for example, retroviral vectors, other lentivirus vectors such as those based on the human immunodeficiency virus (HIV), adenovirus vectors, adeno-associated virus vectors, herpesvirus vectors, vaccinia virus vectors, and the like (see Miller and Rosman, BioTechniques 7: 980-990,1992 ; Anderson et al., Nature 392: 25-30 Suppl., 1998 ; Verma and Somia, Nature 389 : 239-242,1997 ; Wilson, New Engl. J. Med. 334: 1185-1187 (1996), each of which is incorporated herein by reference). Preferred gene transfer vectors are replication-deficient adenovirus carrying thecDNA to effect development of collateral arteries in a subject suffering progressive coronary occlusion(Barr et al., "PCGT Catheter-Based Gene Transfer Into the Heart Using Replication-Deficient RecombinantAdenoviruses,"Journal of Cellular Biochemistry, Supplement 17D, p. 195, AbstractP101 (Mar. 1993); Barr et al. ,"Efficient catheter-mediated gene transfer into the heart using replication-defective adenovirus,"Gene Therapy, vol. 1:51-58 (1994) ). In general, the gene of interest may be transferred to the heart (or skeletal muscle), including

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cardiac myocytes (and skeletal myocytes), in vivo and direct constitutive production of the encoded protein.

[0036] Several different gene transfer approaches are feasible, including the helper-independent replication deficient human adenovirus 5 system. The recombinant adenoviral vectors based on the human adenovirus 5 (Virology 163: 614 617, 1988) are missing essential early genes from the adenoviral genome (usually EIA/E1B), and are therefore unable to replicate unless grown in permissive cell lines that provide the missing gene products in trans. In place of the missing adenoviral genomic sequences, a transgene of interest can be cloned and expressed in tissue/cells infected with the replication deficient adenovirus. Although adenovirus-based gene transfer does not result in integration of the transgene into the host genome (less than 0.1% adenovirus-mediated transfections result in transgene incorporation into host DNA), and therefore is not stable, adenoviral vectors can be propagated in high titer and transfect non-replicating cells well.

[0037] The amount of exogenous nucleic acid introduced into a host organism, cell or cellular system can be varied by those of skill in the art according to the needs of the individual being treated. For example, when a viral vector is employed to achieve gene transfer, the amount of nucleic acid introduced to the cells to be transfected can be varied by varying the amount of plaque forming units (PFU) of the viral vector.

[0038] In yet another embodiment according to the invention, there are provided methods for enhancing collateral blood vessel formation in a subject in need thereof by obtaining ABM from the patient; growing the ABM under suitable culture conditions in a container for a period of time sufficient to promote production by the bone marrow of early attaching cells, which early attaching cells adhere to the container. The early attaching cells are transfected in culture as described above (i. e. in vitro) with a vector as described herein comprising a polynucleotide that encodes one or more agents selected from angiogenic cytokines, growth factors and mammalian angiogenesis-promoting factors, and the like, and the processed (i. e. , transfected) early attaching cells (and/or medium in which they are cultured after transfection) are then directly administered to a desired site in the patient so as to deliver to the site the expressed agent (s). For example,

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in one embodiment, the angiogenesis-promoting agent can be transiently expressed in the subject into which the transfected cells are injected, thus delivering the therapeutic angiogenesis-promoting agents, or

a combination thereof, to the ischemic site and leading to enhanced collateral blood vessel formation at the site of administration in the patient.

[0039] As used herein, the phrase"transcription regulatory region"refers to that portion of a nucleic acid or gene construct that controls the initiation of mRNA transcription. Regulatory regions contemplated for use herein, in the absence of the non- mammalian transactivator, typically comprise at least a minimal promoter in combination with a regulatory element responsive to the ligand/receptor peptide complex. A minimal promoter, when combined with a regulatory element, functions to initiate mRNA transcription in response to a ligand/functional dimer complex. However, transcription will not occur unless the required inducer (ligand therefor) is present. However, as described herein certain of the invention chimeric protein heterodimers activate or repressmRNA transcription even in the absence of ligand for the DNA binding domain.

[0040] As used herein, the phrase"operatively associated with"refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and promoter such that transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA.

[0041] Preferably, the transcription regulatory region further comprises a binding site for ubiquitous transcription factor (s). Such binding sites are preferably positioned between the promoter and the regulatory element. Suitable ubiquitous transcription factors for use herein are well known in the art and include, for example, Spl.

[0042] Exemplary eukaryotic expression vectors include eukaryotic constructs, such as the pSV-2 gpt system (Mulligan et al., (1979) Nature, 277: 108-114); PBLUESKRIPTX vector (Stratagene, La Jolla, CA), the expression cloning vector described by Genetics

Institute (Science, (1985) 228: 810-815), and the like. Each of these plasmid vectors is capable of promoting expression of the protein of interest.

[0043] In a specific embodiment, a gene transfer vector contemplated for use herein is a viral vector, such as Adenovirus, adeno-associated virus, a herpes-simplex virus based vector, a synthetic vector for gene therapy, and the like (see, e. g. , Suhr et al., Arch. of Neurol. 50: 1252-1268,1993). For example, a gene transfer vector employed herein can be a retroviral vector. Retroviral vectors contemplated for use herein are gene transfer plasmids that have an expression construct containing an exogenous nucleic acid residing between two retroviral LTRs. Retroviral vectors typically contain appropriate packaging signals that enable the retroviral vector, or RNA transcribed using the retroviral vector as a template, to be packaged into a viral virion in an appropriate packaging cell line (see, e. g. , U. S. Patent 4,650, 764).

[0044] Suitable retroviral vectors for use herein are described, for example, in U. S.

Patents 5,399, 346 and 5,252, 479; and in WIPO publications WO 92/07573, WO 90/06997, WO 89/05345, WO 92/05266 and WO92/14829, each of which is hereby incorporated herein by reference, in its entirety. These documents provide a description of methods for efficiently introducing nucleic acids into human cells using such retroviral vectors. Other retroviral vectors include, for example, mouse mammary tumor virus vectors (e. g. , Shackleford etal., (1988) PNAS, USA, 85: 9655-9659), human immunodeficiency virus (e. g. , Naldini et al. (1996) Science 272: 165-320), and the like.

[0045] Various procedures are also well known in the art for providing helper cells that produce retroviral vector particles that are essentially free of replicating virus. See, for example, U. S. Patent 4,650, 764; Miller, Human Gene Therapy, 1 : 5-14, 1990; Markowitz, et al., Journal of Virology, 61 (4 ! : 1120-1124, 1988 ; Watanabe, et al., Molecular and Cellular Biology, 3 12 : 2241-2249,1983 ; Danos, et al., PNAS, 85 : 6460- 6464, 1988 ; and Bosselman, et al., Molecular and Cellular Biology, 7 (5 ! : 1797-1806, 1987, which disclose procedures for producing viral vectors and helper cells that minimize the chances for producing a viral vector that includes a replicating virus.

[0046] Recombinant retroviruses suitable for prepackaging with polynucleotides that encode therapeutic proteins, such as angiogenic growth factors, are produced employing well-known methods for producing retroviral virions. See, for example, U. S. Patent 4,650, 764; Miller, supra 1990; Markowitz, et al., supra 1988; Watanabe, et al., supra 1983; Danos, et al., PNAS, 85 : 6460-6464,1988 ; and Bosselman, et al., Molecular and CellularBiology, 7 (5 ! : 1797-1806, 1987.

[0047] In the examples below, certain testing regarding aspects of the invention is set forth. These examples are non-limitative.

EXAMPLES
EXAMPLE 1 Effect of Bone Marrow Cultured Media-on Endothelial Cell Proliferation[0048] Studies were conducted to determine whether aspirated pig autologous bone marrow cells obtained secreted VEGF, a potent angiogenic factor, and MCP-1, which recently has been identified as an important angiogenic co-factor. Bone marrow was cultured in vitro for four weeks. The conditioned medium was added to cultured pig aortic endothelial cells (PAECs), and after four days proliferation was assessed. VEGF andMCP-1 levels in the conditioned medium were assayed using ELISA. During the four weeks in culture, BM cells secreted VEGF and MCP-1, such that their concentrations increased in a time-related manner. The resulting conditioned medium enhanced, in a dose-related manner, the proliferation of PAECs. The results indicate that BM cells are capable of secreting potent angiogenic cytokines such as VEGF and MCP-1 and of inducing proliferation of vascular endothelial cells.

Pig Bone Marrow Culture[0049] Bone marrow (BM) cells were harvested under sterile conditions from pigs with chronic myocardial ischemia in preservative free heparin (20 units/ml BM cells) and filtered sequentially using30011 and200p stainless steel mesh filters. BM cells were then isolated by Ficoll-Hypaque gradient centrifugation and cultured in long-term culture medium (LTCM) (Stem Cell Tech, Vancouver, British Columbia, Canada) at330 C with

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5% C02 in T-25 culture flask. The seeding density of the BMCs in each culture was 7 x 106/ml. Weekly, one half of the medium was removed and replaced with fresh LTCM.

The removed medium was filtered (0.2L filter) and storedat-200 C for subsequent Enzyme-linkedImmunosorbent Assay (ELISA) and cell proliferation assays.

Isolation and Culture of Pig Aortic Endothelial Cells[0050] Fresh pig aortic endothelial cells (PAECs) were isolated using conventional methods. Endothelial cell growth medium (EGM-2 medium, Clonetics, San Diego, CA), containing 2% FBS, hydrocortisone, human FGF, VEGF, human EGF, IGF, heparin and antibiotics, at37 C with 5% carbon dioxide. When the cells became confluent at about 7 days, they were split by 2.5% trypsin and cultured thereafter in medium 199 with 10% FBS. Their identity was confirmed by typical endothelial cell morphology and by immunohistochemistry staining for factor VIII. Passage 3-10 was used for the proliferation study.

Effects of conditioned medium on aortic endothelial cells[0051] Cell proliferation assay: PAECs (Passage 3-10) were removed from culture flasks by trypsinization. The detached cells were transferred to 96-well culture plates and plated at a seeding density of 5,000 cells/well. Cells were cultured for 2-3 days before being used in proliferation and DNA synthesis experiments. The conditioned medium of BM cells cultures were collected at 4 weeks, medium from 7 culture flasks were pooled and used in the bioassay. Aliquotes (10pL, 30L, 100nL or200 gel) of pooled conditioned medium, or LTCM(200, uL, as control), were added to confluent PAECs in 96-well plates in triplicate. Four days following culture with conditioned medium or control medium, the PAECs were trypsinized and counted using a cell counter (Coulter Counter Beckman Corporation, Miami FL).

Effects of Conditioned Medium onPAEC DNA Synthesis[0052] Aliquotes (10 uL, 30pL, 100all or 200p1L) of conditioned medium from pooled samples or control medium (LTCM,200, ut) were added to PAECs in 96-well plate (same seeding density as above) in triplicate. After 2 days,1 jj, Ci tritiated thymidine was added to each well. Forty-eight hours later, DNA in PAECs was harvested using a

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cell harvester (Mach III M Tomtec, Hamden, CT) and radioactivity was counted by liquid scintillation counter (Multi-detector Liquid Scintillation Luminescence Counter EG & G Wallac, Turku, Finland).

Determination of VEGF andMCP-1 in conditioned medium by ELISA VEGF[0053] The concentration of VEGF in conditioned medium was measured using a sandwich ELISA kit (Chemicon International Inc. , Temecula, CA). Briefly, a plate pre- coated with anti-human VEGF antibody was used to bind VEGF in the conditioned medium or to a known concentration of recombinant VEGF. The complex was detected by the biotinylatedanti-VEGF antibody, which binds to the captured VEGF. The biotinylated VEGF antibody in turn was detected by streptavidin-alkaline phosphatase and color generating solution. The anti-human VEGF antibody cross-reacts with porcine VEGF.

Determination ofMCP-1 in conditioned medium by ELISA[0054] The concentration of MCP-1 in conditioned medium was assayed by sandwich enzyme immunoassay kit (R & D Systems, Minneapolis, MN): a plate pre-coated with anti human MCP-1 antibody was used to bind MCP-1 in the conditioned medium or to a known concentration of recombinant protein. The complex was detected by the biotinylatedanti-MCP-1 antibody, which binds to the captured MCP-1. The biotinylated MCP-1 antibody in turn was detected bystreptavidin-alkaline phosphatase and color generating solution. The anti-humanMCP-1 antibody cross-reacts with porcine MCP-1.

Results[0055] The BM conditioned medium collected at four weeks increased, in a dose- related manner, the proliferation of PAECs (Fig. 1). This was demonstrated by counting the number of cells directly and by measuring tritiatedthymidine uptake($p < 0.001$ for both measurements). The dose-related response demonstrated a descending limb; proliferation decreased with 200 uL conditioned medium compared to 30uL and100 , L ($P = 0.003$ for both comparisons). Similar dose-related results were observed in the tritiatedthymidine uptake studies ($P = 0.03$ for 30uL and 100 pL compared to 200uL, respectively).

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[0056] A limited number (5 i 4%) of freshly aspirated BM cells stained positive for factor VIII. The results are set forth in Fig. 2. This contrasted to 57 A 14% of the adherent layer of BM cells cultured for 4 weeks, of which 60 23% were endothelial- like cells and 40 i 28% appeared to be megakaryocytes.

[0057] Over a 4-week period, the concentrations of VEGF and MCP-1 in the BM conditioned medium increased gradually to 10 and 3 times the 1st week level, respectively ($P < 0.001$ for both comparisons) (Fig. 3). In comparison, VEGF and MCP-1 levels in a control culture medium, not exposed to BM, were 0 and 11 i 2 pg/ml, respectively, as shown in Fig. 4.

EXAMPLE 2 Effects of Hypoxia on VEGF Secretion by Cultured Pig Bone Marrow Cells[0058] It was demonstrated that hypoxia markedly increases the expression of VEGF by cultured bone marrow endothelial cells, results indicating that ex-vivo exposure to hypoxia, by increasing expression of hypoxia-inducible angiogenic factors, can further increase the collateral enhancing effect of bone marrow cells and its conditioned media to be injected in ischemic muscular tissue. Pig bone marrow was harvested and filtered sequentially using30011 and 20011 stainless steel mesh filters. BMCs were then isolated by Ficoll-Hypaque gradient centrifugation and cultured at33 C with 5% COx in T-75 culture flasks. When cells became confluent at about 7 days, they were split 1: 3 by trypsinization. After 4 weeks of culture, the BMCs were either exposed to hypoxic conditions (placed in a chamber containing1% oxygen) for 24 to 120 hrs, or maintained under normal conditions. The resulting conditioned medium was collected and VEGF,MCP-1 were analyzed by ELISA.

[0059] Exposure to hypoxia markedly increased VEGF secretion: At 24 hours VEGF concentration increased from 106 + 13 pg/ml under normoxic, to 1,600 i 196 pg/ml under hypoxic conditions ($p = 0.0002$) ; after 120 hours it increased from 4,163 i 62 to 6,028
167 pg/ml ($p < 0.001$). A separate study was performed on freshly isolated BMCs, and the same trend was found. Hypoxia also slowed the rate of proliferation of BMCs. MCP-1

expression was not increased by hypoxia, a not unexpected finding as its promoter is not known to have HIF binding sites.

EXAMPLE 3 Effect of Bone Marrow Cultured Media on Endothelial Cell Tube Formation[0060] It was demonstrated, using pig endothelial cells and vascular smooth muscle cells co-culture technique, that the conditioned medium of bone marrow cells induced the formation of structural vascular tubes in vitro. No such effect on vascular tube formation was observed without exposure to bone marrow conditioned medium. The results suggest that bone marrow cells and their secreted factors exert pro-angiogenic effects.

EXAMPLE 4 The effect of Transendocardial Delivery of Autologous Bone Marrow on Collateral Perfusion and Regional Function in Chronic Myocardial Ischemia Model[0061] Chronic myocardial ischemia was created in 14 pigs by the implantation of ameroid constrictors around the left circumflex coronary artery. Four weeks after implantation, 7 animals underwent transendocardial injections of freshly aspirated ABM into the ischemic zone using a transendocardial injection catheter (2.4 ml per animal injected at 12 sites) and 7 control animals were injected with heparinized saline. At baseline and 4 weeks later, animals underwent rest and pacing echocardiogram to assess regional contractility (% myocardial thickening), and microsphere study to assess collateral-dependent perfusion at rest and during adenosine infusion. Four weeks after injection of ABM collateral flow (expressed as the ratio of ischemic/normal zone x 100) improved in ABM-treated pigs but not in controls (ABM: 95: : L 13 vs. 81i 11 at rest, P- 0.017 ; 85 19 vs. 72 10 during adenosine, P=0.046 ; Controls: 86i 14 vs. 86 14 at rest, P=NS; 73 1 vs. 72zt 14 during adenosine, P=0.63). Similarly, contractility increased in ABM-treated pigs but not in controls (ABM: 83 21 vs. 60 32 at rest, P=0.04 ;91 i 44 vs. 35 ~ 43 during pacing, P=0.056 ; Controls: 69 48 vs. 64 46 at rest, P=0.74 ;65 56 vs.37 i 56 during pacing, P=0.23).

[0062] The results indicate that catheter-based transendocardial injection of ABM can augment collateral perfusion and myocardial function in ischemic myocardium, findings

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suggesting that this approach may constitute a novel therapeutic strategy for achieving optimal therapeutic angiogenesis.

[0063] Fourteen specific-pathogen-free domestic pigs weighing approximately 70 kg were anesthetized, intubated, and received supplemental 02 at 2 L/min as well as 1-2% isoflurene inhalation throughout the procedure. Arterial access was obtained via right femoral artery isolation and insertion of an 8 French sheath. The left circumflex artery was isolated through a left lateral thoracotomy and a metal encased ameroid constrictor was implanted at the very proximal part of the artery. Four weeks after the ameroid constrictor implantation all pigs underwent (1) a selective left and right coronary angiography for verification of ameroid occlusion and assessment of collateral flow; (2) transthoracic echocardiography studies; and (3) regional myocardial blood flow assessment.

Bone Marrow Aspiration and Preparation andIntramyocardial Injection[0064] Immediately after completion of the baseline assessment, all animals underwent BM aspiration from the left femoral shaft using standard techniques. BM was from aspirated 2 sites (3 ml per site) using preservative free heparinized glass syringes (20 unit heparin/1 ml fresh BM). The aspirated bone marrow was immediately macro-filtered using30011and 200, u stainless steel filters, sequentially. Then, the bone marrow was injected using a transendocardial injection catheter into the myocardium in 12 sites (0.2 ml per injection site for total of 2.4 ml) directed to the ischemic myocardial territory and its borderline region.

Echocardiographyy [0065] Transthoracic echocardiography images of short and long axis views at the mid-papillary muscle level were recorded in animals at baseline and during pacing, at baseline and during follow-up evaluation at four weeks after ABM implantation.

Fractional shortening measurements were obtained by measuring the % wall thickening (end-systolic thickness minus end-diastolicthickness/end-diastolic thickness) x 100.

Those measurements were taken from the ischemic territory (lateral area) and remote territory (anterior-septal area). Subsequently, a temporary pacemaker electrode was

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inserted via a right femoral venous sheath and positioned in the right atrium. Animals were paced at 180/minute for 2 minutes and echocardiographic images were simultaneously recorded.

Regional Myocardial Blood Flow [0066] Regional myocardial blood flow measurements were performed at rest and during maximal coronary vasodilation by use of multiple fluorescent colored microspheres (Interactive Medical Technologies, West Los Angeles, CA) and quantified by the reference sample technique (Heymann MA, et al., Prog CardiovascDis 1977 ; 20: 55-79). Fluorescent microspheres (0.8 ml,5x106 microspheres/ml, 15llm diameter in a saline suspension with 0.01% Tween 80) were injected into the left atrium via a 6F Judkins left 3. 5 diagnostic catheter. Maximal coronary vasodilation was induced by infusing adenosine at a constant rate of140 ug/kg/min (Fujisawa USA, Deerfield, IL) into the left femoral vein over a period of 6 minutes. During the last 2 minutes of the infusion, microsphere injection and blood reference withdrawal were undertaken in identical fashion to the rest study.

[0067] Following completion of the perfusion assessment, animals were sacrificed with an overdose of sodium pentobarbital and KCL. Hearts were harvested, flushed with Ringer Lactate, perfusion-fixed for 10-15 minutes, and subsequently immersion-fixed with 10% buffered formaldehyde for 3 days. After fixation was completed, the hearts were cut along the short axis into 7-mm thick slices. The 2 central slices were each divided into 8 similar sized wedges, which were further cut into endocardial and epicardial subsegments. The average of 8 lateral ischemic zone and 8 septal normal zone sub-segments measurements were used for assessment of endocardial and epicardial regional myocardial blood flow. The relative collateral flow was also computed as the ratio of the ischemic zone/non ischemic zone (IZ/NIZ) blood flow.

Histopathology[0068] To assess whether injecting BM aspirate via the use of an injection catheter was associated with mechanical cell damage, standard BM smears were prepared before and after propelling the freshly filtered ABM aspirate through the needle using similar

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injecting pressure as in the in-vivo study. Morphological assessment was performed by an independent experienced technician who was blinded to the study protocol.

[0069] Histopathology assessment was performed on sampled heart tissue. In the pilot study, 7-mm thick short-axis slices were examined under UV light to identify fluorescent-tagged areas. Each identified area was cut into 3 full thickness adjacent blocks (central, right and left) that were immersion-fixed in 10% buffered formaldehyde.

Subsequently, each such block was cut into 3 levels, of which 2 were stained with Hematoxylin and Eosin (H & E) and one with PAS. In addition, one fresh fluorescent- labeled tissue block was obtained from the ischemic region of each animal and was embedded in OCT compound (Sakura Finetek USAInc., Torrance, CA) and frozen in liquid nitrogen. Frozen sections of these snap-frozen myocardial tissues were air dried and fixed with acetone. Immunoperoxidase stain was performed with the automated Dako immunno Stainer (Dako, Carpenteria, CA). The intrinsic peroxidase and non- specific uptake were blocked with 0.3% hydrogen peroxidase and 10% ovo-albumin.

Monoclonal mouse antibody against CD-34 (Becton Dickinson, San Jose, CA) was used as the primary antibody. The linking antibody was a biotinylated goat anti-mouse IgG antibody and the tertiary antibody was strepavidin conjugated with horse reddish peroxidase. Diaminobenzidine (DAB) was used as the chromogen and the sections were counterstained with 1% methylgreen. After dehydration and clearing, the slides were mounted and examined with a Nikon Labphot microscope.

[0070] In the efficacy study, full-thickness, 1.5 square centimeter sections from the ischemic and non-ischemic regions were processed for paraffin sections. Each of the samples was stained with H & E, Masson's trichrome, and factor VIII related antigen. The immunoperoxidase stained slides were studied for density of endothelial cell population and vascularization. The latter was distinguished from the former by the presence of a lumen. Vascularity was assessed using 5 photomicrographs samples of the factor VIII stained slides taken from the inner half of the ischemic and non-ischemic myocardium.

Density of endothelial cells was assessed using digitized images of the same photomicrographs. The density of the endothelial population was determined by Sigma- Scan Pro morphometry software using the

intensity threshold method. The total

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endothelial area for each sample as well as for each specimen were obtained along with the relative percent endothelial area (endothelial area/area of the myocardium studied).

The total endothelial area was also calculated as the relative percent of the non-infarcted (viable) area of the myocardium studied. The trichrome stained sections were digitized and the area occupied by the blue staining collagen as well as the total area of the section excluding the area occupied by the epicardium (which normally contained collagen) were measured using Sigma-Scan Pro. The infarcted area was then calculated as the area occupied by the blue staining.

Procedural Data[0071] Intra-myocardial injections either with ABM or placebo were not associated with any acute change in mean blood pressure, heart rate or induction of arrhythmia. All hemodynamic parameters were comparable between the two groups. Pair-wise comparison showed similar hemodynamic parameters within each group in the index compared to the follow-up procedure except for higher initial mean arterial blood pressure at follow-up in the control group (P=0.03) with no subsequent differences during pacing or adenosine infusion.

Myocardial Function[0072] Regional myocardial function assessment is shown in Table I below.

Preintervention relative fractional wall thickening, expressed as ischemic zone to non- ischemic zone (IZ/NIZ) ratio x 100, at rest and during pacing, was similar between groups (P=0.86 and 0.96, respectively). At-4 weeks following the intra-myocardial injection of ABM, improved regional wall thickening occurred at rest and during pacing, which was due toan-50% increase in wall thickening of the collateral-dependent ischemic lateral wall. No significant changes were observed in the control animals, although a trend towards improvement in wall thickening was noted in the ischemic area during pacing at follow-up.

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Tablel. RegionalContractility of the Ischemic Wall Baseline Follow-up P
Rest
ABM(%) 60 i 32 83 i 21 0.04
Control(%) 64 46 69 48 0.74
Pacing
ABM (%) 36 ~ 43 91 44 0.056
Control (%) 37 ~ 56 65 ~ 56 0.23 ABM indicates autologous bone marrow.

Myocardial Perfusion Data[0073] Regional myocardial perfusion assessment is shown in Table II below. There were no differences between the treated and control groups in the pre-intervention relative transmural myocardial perfusion, IZ/NIZ, at rest and during adenosine infusion (P=0. 42 and 0.96, respectively). At 4 weeks following ABM injection, relative regional transmural myocardial perfusion at rest and during pacing improved significantly. This was due to an absolute improvement in myocardial perfusion in the ischemic zone both at rest (an increase of 57%, P=0. 08) and during adenosine infusion (37%, P=0.09), while no significant changes were noted in absolute flow to the non-ischemic zone either at rest (increase of 35%, P=0. 18) or during adenosine infusion (increase of 25%, P=0.26). The increase in regional myocardial blood flow found in the ischemic zones consisted of both endocardial (73%) and epicardial (62%) regional improvement at rest, with somewhat lesser improvement during adenosine infusion (40% in both zones). At 4 weeks, the control group showed no differences in transmural, endocardial or epicardial perfusion in the ischemic and non-ischemic zones compared to pre-intervention values.

Table II. Regional Myocardial Perfusion Baseline Follow-up P
Rest
ABM (%)8312 9814 0.001
Control (%) 89 ~ 9 92 ~ 0. 1 0.43
Adenosine

ABM (%)788918 0.025
Control (%)77578110. 75 ABM indicates autologous bone marrow.

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Histopathology and Vascularity Assessment[0074] Assessment of BM smears before and after passing the filtrated aspirate through the injecting catheter revealed normal structure, absence of macro-aggregates and no evidence of cell fragments or distorted cell shapes. Histopathology at day 1 following injections revealed acute lesions characterized by fibrin and inflammatory tract with dispersed cellular infiltration. The infiltrate was characterized by mononuclear cells that morphologically could not be differentiated from a BM infiltrate. Cellularity was maximal at 3 and 7 days and declined subsequently over time. At 3 weeks, more fibrosis was seen in the 0.5 ml injection-sites compared to the 0.2 ml. CD-34 immunostatining, designed to identify BM-derived progenitor cells, was performed in sections demonstrating the maximal cellular infiltrate. Overall, it was estimated that 4-6% of the cellular infiltrate showed positive immunoreactivity to CD-34.

[0075] Small areas of patchy necrosis occupying overall < 10% of the examined ischemic myocardium characterized the ischemic territory in both groups. The non- ischemic area revealed normal myocardial structure. Changes in the histomorphometric characteristics of the two groups were compared. There were no differences in the total area occupied by any blood vessel as well as the number of blood vessels > 50 um in diameter. However, comparison of the total areas stained positive for factor VIII (endothelial cells with and without lumen) in the ischemic versus the non-ischemic territories revealed differences between the 2 groups. In the ABM group, the total endothelial cell area in the ischemic collateral-dependent zone was 100% higher than that observed in the nonischemic territory (11.6i 5.0 vs. 5.7i 2.3% area, P=0.016), whereas there was no significant difference in the control group (12.3A 5.5 vs. 8.2i 3.1% area,P=0. 11). However, other parameters of vascularity, including % area occupied by any blood vessel and number of blood vessels >50 lem were similar in the ischemic and non- ischemic territories in both groups.

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EXAMPLE 5 The effect of autologous bone marrow stimulated in vivo by pre-administration of GM- CSF in animal model of myocardial ischemia[0076] Chronic myocardial ischemia was created in 16 pigs by the implantation of ameroid constrictors around the left circumflex coronary artery. At four weeks minus 3 days after ameroid implantation, 8 animals underwent subcutaneous injection of GM-CSF for 3 consecutive days (dose10 u, g per day) followed (on the fourth day and exactly 4 weeks after ameroid implantation) by transendocardial injections of freshly aspirated ABM into the ischemic zone using a transendocardial injection catheter (2.4 ml per animal injected at 12 sites) and8 control animals without GM-CSF stimulation were injected with heparinized saline. At baseline and 4 weeks later, animals underwent rest and pacing echocardiogram to assess regional contractility (% myocardial thickening), and microsphere study to assess collateral-dependent perfusion at rest and during adenosine infusion. Four weeks after injection of ABM collateral flow (expressed as the ratioof ischemic/normal zone x 100) improved in ABM-treated pigs but not in controls (ABM:85- 11 vs. 72A 16 at rest, P=0.026 ; 83i 18 vs. 64 19 during adenosine, P=0.06 ; Controls: 93 10 vs. 89 9 at rest, P=0.31 ; 73zt 17 vs. 75i 8 during adenosine, P=0.74). Similarly, contractility increased in ABM-treated pigs but not in controls (ABM: 93i 33 vs. 63A 27 at rest, P=0.009 ;84 i 36 vs. 51i 20 during pacing, P=0.014, Controls: 72i 45 vs. 66 43 at rest, P=0.65 ; 70i 36 vs. 43i 55 during pacing,P=0.18).

[0077] The results indicate that catheter-based transendocardial injection of ABM prestimulated in vivo by GM-CSF administered systemically for 3 days, can augment collateral perfusion and myocardial function in ischemic myocardium, findings suggesting that this approach may constitute a novel therapeutic strategy for achieving optimal therapeutic angiogenesis.

EXAMPLE 6 Treatment of a Human Patient[0078] Bone marrow (-5 ml) will be aspirated from the iliac crest at approximately 60 minutes prior to initiation of the cardiac procedure using preservative-free heparinized glass syringes (20 unit heparin/1 ml fresh BM). The aspirated bone marrow will be

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immediately macro-filtered using300p and200 stainless steel filters, sequentially. An experienced hematologist will perform the procedure under sterile conditions. The bone marrow smear will be evaluated to confirm a normal histomorphology of the bone marrow preparation.

[0079] Any of several procedures for delivery of an agent to the myocardium can be used. These include direct transepicardial delivery, as could be achieved by a surgical approach (for example, but not limited to, a transthoracic incision or transthoracic insertion of a needle or other delivery device, or via thoracoscopy), or by any of several percutaneous procedures. Following is one example of percutaneous delivery. It should be emphasized that the following example is not meant to limit the options of delivery to the specific catheter-based platform system described in the example-any catheter-based platform system can be used.

[0080] Using standard procedures for percutaneous coronary angioplasty, an introducer sheath of at least SF is inserted in the right or left femoral artery. Following insertion of the arterial sheath, heparin is administered and supplemented as needed to maintain an ACT for 200-250 seconds throughout the LV mapping and ABM transplantation portion of the procedure. ACT will be checked during the procedure at intervals of no longer than 30 minutes, as well as at the end of the procedure to verify conformity with this requirement.

[0081] Left ventriculography is performed in standard RAO and/or LAO views to assist with guidance of NOGA-START and injection catheters, and an LV electro- mechanical map is obtained using the NOGA-STAR catheter. The 8F INJECTION- STAR catheter is placed in a retrograde fashion via the femoral sheath to the aortic valve.

After full tip deflection, the rounded distal tip is gently prolapsed across the aortic valve and straightened appropriately once within the LV cavity.

[0082] The catheter (incorporating an electromagnetic tip sensor) is oriented to one of the treatment zones (e. g. , anterior, lateral, inferior-posterior or other). Utilizing the safety features of the NOGA TM system, needle insertion and injection is allowed only when stability signals will demonstrate an LS value of < 3. A single injection of 0.2 cc of

freshly aspirated ABM will be delivered via trans-endocardial approach to the confines of up to two treatment zones with no closer than 5 mm between each injection site. The density of injection sites will depend upon the individual subject's LV endomyocardial anatomy and the ability to achieve a stable position on the endocardial surface without catheter displacement or premature ventricular contractions (PVCs).

[0083] That freshly aspirated ABM transplanted into ischemic myocardium is associated with improved collateral flow without adverse effects may be of clinical importance for several reasons. The methodology reflected above took advantage of the natural capability of the bone marrow to induce a localized angiogenic response in an effective and apparently safe manner. Such an angiogenic strategy would probably be less costly than many others currently being tested. It would also avoid potential toxicity-related issues that are remote but definite possibilities with various gene-based approaches using viral vectors.

[0084] The invention is based on the concept that ABM may be an optimal source for cellular (an example would be endothelial progenitor cells, but the invention is not limited to such cells as many other cells in the bone marrow may contribute importantly to the angiogenic effect) and secreted, e. g., angiogenic growth factors, elements necessary to promote new blood vessel growth and restore function when transferred to another tissue, such as ischemic heart or peripheral limbs. A patient's own bone marrow can be used as the key therapeutic source to induce therapeutic angiogenesis and/or myogenesis in ischemic tissues, e. g. , heart muscle and/or ischemic limb, with compromised blood perfusion due to arterial obstructions. The patient's own bone marrow is aspirated, i. e., ABM donation, processed as described herein, and injected directly into ischemiaand/or adjacent non-ischemic tissue, e. g. , heart muscleand/or ischemic limb, to promote blood vessel growth.

[0085] The ABMand/or bone marrow products are injected into the heart muscle, e. g., the myocardium, by

use of either a catheter-based trans-endocardial injection approach or a surgical (open chest or via thoracoscopy) trans-epicardial thoracotomy approach. Those two delivery strategies can be used to achieve the same therapeutic goal by promoting the

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incorporation and integration of angiogenic bone marrow elements in the target organ tissue, e. g. , heart muscle and/or ischemic limb.

[0086] According to the invention, effective amounts of ABM, bone marrow cells or bone marrow cells transfected with an angiogenesis-promoting agent are administered for treatment. As would be appreciated by experienced practitioners, the amount administered will depend upon many factors, including, but not limited to, the intended treatment, the severity of a condition being treated, the size and extent of an area to be treated, etc. With regard to treatment according to the invention, a representative protocol would be to administer quantities of from about 0.2 to about 0.5 ml of ABM in each of from about 12 to about 25 injections, for a total of from about 2.4 to about 6 ml of ABM being administered. Each dose administered could preferably comprise from about 1 to about 2 percent by volume of heparin or another blood anticoagulant, such as coumadin.

When the ABM has been cultured or stimulatedand/or is being administered in combination with other pharmaceuticals or the like, the quantity of ABM present should be approximately the same in each dose and/or the total of the ABM administered should be about the same as described above. It is believed that the total number of cells of ABM administered in each treatment should be on the order of from about107 to5X108.

[0087] In another embodiment of the invention, optimization of angiogenic gene expression may be enhanced by co-administration of various angiogenic stimulants with the ABM. Thus, according to the invention ABM transplantation is injected either as a "stand alone"therapeutic agent or combined with any pharmacologic drug, protein or gene or any other compound or intervention that may enhance bone marrow production of angiogenic growth factors and/or promote endothelial cell proliferation, migration, and blood vessel tube formation. The"combined"agent (s) can be administered directly into the patient or target tissue, or incubated ex-vivo with bone marrow prior to injection of bone marrow into the patient. Examples of these"combined"agents (although not limited to these agents) are Granulocyte-Monocyte Colony Stimulatory Factor (GM- CSF), Monocyte Chemoattractant Protein 1 (MCP 1), EPAS1, or Hypoxia Inducible Factor-1(HIF-1). The stimulation of the bone marrow could be by the direct exposure of the bone marrow to the factors in the form of proteins, or the bone marrow cells can be

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transfected with vectors carrying the relevant genes. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes. An example of an intervention that may enhance bone production of angiogenic factors is ex-vivo exposure of bone marrow cells to hypoxia. This intervention can be used alone with bone marrow, or in combination with any of the factors outlined above. These optimization strategies are designed to increase the production of vascular endothelial growth factor (VEGF) expression and/or other cytokines with angiogenic activity prior to the direct injection of the bone marrow into the heart or any peripheral ischemic tissue. In a broad sense, the invention comprises intramyocardial injection of ABM with any agent that would become available to cause stimulation of bone marrowand/or ex-vivo or in vivo stimulation of any angiogenic growth factor production by the bone marrow or its stromal microenvironment.

[0088] Delivery of the above-described therapeutic modalities to patients will vary, dependent upon the clinical situation. For example, patients with refractory coronary artery disease or ischemic peripheral vasculopathy will be candidates for a bone marrow aspiration procedure followed by ABM myocardial or limb transplantation directed into the ischemic tissue or its borderline zone and/or normal tissue that may serve as the source for collateral or cellular supply to the diseased tissue for the purposes of therapeutic angiogenesis and/or myogenesis. For example, patients with refractory coronary artery disease or ischemic peripheral vasculopathy will be candidates for a bone marrow aspiration procedure followed by ABM myocardial or limb transplantation directed into the ischemic tissue or its borderline zoneand/or normal tissue that may serve as the source for collateral or cellular supply to the diseased tissue for the purposes of therapeutic angiogenesisand/or myogenesis. This procedure will involve the use of a bone marrow aspiration procedure, bone marrow harvesting and processing, followed by the use of the ABM or

its elements (growth factorsand/or cellular elements being isolated from the patient's own bone marrow), with or without any ex-vivo stimulation of its delivery forms, to be injected into the ischemic or non ischemic myocardium and/or peripheral ischemic tissue (such as limb ischemia). The bone marrow will be kept in standard anticoagulation/anti-aggregation solution (containing sodium citrate and EDTA) and kept in4 C in sterile medium until the time of its use.

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[0089] Upon its use, the bone marrow will be filtered to avoid injecting remaining blood clots or macroaggregates into the target tissue.

[0090] The bone marrow, with or without a stimulatory agent in any of its delivery forms, or with or without having been transfected with a vector carrying a transgene that is designed to enhance the angiogenesis effect of the bone marrow, will be injected into the heart muscle, i. e. , in therapeutic myocardial angiogenesis or therapeutic myogenesis, using either any catheter-based trans-endocardial injection device or via a surgical (open chest) trans-epicardial thoracotomy approach, or any other approach that allows for transepicardial delivery. In the case of treatment of limb ischemia the bone marrow will be transferred by a direct injection of the bone marrow or it elements, with or without ex- vivo or in vivo stimulation in any of its delivery forms, into the muscles of the leg.

[0091] The volume of injection per treatment site will probably range between 0.1-5. 0 cc per injection site, dependent upon the specific bone marrow product and severity of the ischemic condition and the site of injection. The total number of injections will probably range between 1-50 injection sites per treatment session.

EXAMPLE 7 Pig Bone Marrow Culture[0092] Bone marrow cells(BMCs) are harvested under sterile conditions from pigs in preservative free heparin (20 units/ml BM cells) and filtered sequentially using30OR and20OR stainless steel mesh filters. BMCs are then isolated by Ficoll-Hypaque gradient . centrifugation, seeded in T-75 flasks, and cultured overnight in long-term culture medium (LTCM) (Stem Cell Tech, Vancouver, British Columbia, Canada) at33 C with 5% C02 in T-75 culture flasks. The medium is then changed and the non-attaching cells washed out. The attached cells (i. e. , early attaching cells) are mostly monocytes, endothelial precursor cells, or other hemopoietic lineage cells. By lac-Z staining testing, these cells have been shown to be permissive for adenovirus by expression of the marker protein.

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[0093] The seeding density of the BMCs in each culture dish is 7 x 106/ml. When the cells become confluent at about 7 days, they are split 1 to 3 by 0.25% trypsin. Passages 3-8 were used for this study.

Adenovirus Transfection[0094] BMCs are first cultured in 6-cm Petri dishes for 3 to 14 days to allow for production of a lining of early attaching cells that adhere to the Petri dish. The non- adherent cells are washed away the day after initial seeding. Then the early attaching cells are inoculated with a vector encoding one or more cytokines, growth factors, or other mammalian angiogenesis promoting factors, such as, but not limited to, the transcription factorsHIP-1 or HIF-2. This inoculation can occur from 3 to28 days after seeding. This virus is washed out from the transfected cells about 2 hours to 3 days after inoculation. The transfected cells can then be injected into the patient's target tissue, such as the muscle of heart or leg. The present invention may be embodied in other specific forms without departing from the spirit or central attributes thereof. Thus, the foregoing description of the present invention discloses only exemplary embodiments thereof, and other variations are contemplated as being within the scope of the present invention.

Accordingly, the present invention is not limited to the particular embodiments that have been described in detail herein. Rather, reference should be made to the appended claims as indicative of the scope and content of the invention.

[0095] The preceding specific embodiments are illustrative of the practice of the invention. It is to be understood, however, that other expedients known to those skilled in the art or disclosed herein, may be

employed without departing from the spirit of the invention or the scope of the appended claims.

# INTRAMYOCARDIAL INJECTION OF AUTOLOGOUS BONE MARROW

Claims of **WO03101201**

WHAT IS CLAIMED IS:1. A method for enhancing transfection efficiency in bone marrow cells, said method comprising: growing bone marrow cells under suitable culture conditions in a container for a period of time sufficient to promote production by the bone marrow of early attaching cells; and transfecting the early attaching cells with a vector comprising a polynucleotide that encodes one or more agents selected from angiogenic cytokines, growth factors and mammalian angiogenesis-promoting factors, thereby enhancing transfection efficiency in the early attaching cells as compared with that obtained by transfecting fresh bone marrow or non-adherent bone marrow cells with the vector.

2. The method of claim 1, wherein the period of time is from about 3 days to about28 days.

3. The method of claim 1, wherein the period of time is from about 3 days to about 14 days.

4. The method of claim1, wherein the period of time is from about 3 days to about 7 days.

5. The method of claim 1, wherein the bone marrow is filtered.

6. The method of claim 5, wherein the filtering removes particles larger than from about300g to about2001l.

7. The method of claim 1, wherein the one or more agents are selected fromHIF-1, EPAS1, MCP-1, and CM-CSF.

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8. The method of claim 1, wherein the vector is selected from a plasmid vector and an adenoviral vector.

9. The method of claim 8, wherein the vector is an adenoviral vector.

10. The method of claim 1, further comprising stimulating the transfected early attaching cells.

11. The method of claim 10, wherein the mammalian angiogenesis-promoting factor is a transcription factor.

12. The method of claim 11, wherein the transcription factor is HIF-1.

13. A method for enhancing collateral blood vessel formation in a patient in need thereof, said method comprising: obtaining autologous bone marrow from the patient; growing the autologous bone marrow under suitable culture conditions in a container for a period of time sufficient to promote production by the bone marrow of early attaching cells; transfecting the ear y attaching cells with a vector comprising a polynucleotide that encodes one or more agents selected from angiogenic cytokines, growth factors and mammalian angiogenesis-promoting factors so as to cause expression of the one or more agents; and directly administering to a desired site in the patient an effective amount of the transfected early attaching cells, thereby enhancing collateral blood vessel formation at the site in the patient.

14. The method of claim 13, wherein the period of time is from about 3 days to about 28 days.

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15. The method of claim 13, wherein the period of time is from about 3 days to about 14 days.

16. The method of claim 13, wherein the period of time is from about 3 days to about 7 days.

17. The method of claim 13, further comprising filtering the autologous bone marrow prior to placement in the container.

18. The method of claim 17, wherein the filtering removes particles larger than from about300t to about200jan.

19. The method of claim 13, wherein the one or more agents are selected from HIF-1,EPAS 1, MCP-1, and CM-CSF.

20. The method of claim 13, wherein the mammalian angiogenesis-promoting factor is a transcription factor.

21. The method of claim 13, wherein the agent isHIF-1 and the vector is an adenoviral vector.

22. The method of claim 13, wherein the vector is selected from a plasmid vector and an adenoviral vector.

23. The method of claim 22, wherein the vector is an adenoviral vector.

24. The method of claim 13, wherein the autologous bone marrow is processed to obtain therefrom bone marrow cells for placement in the container.

25. The method of claim 13, wherein the transfected cells are injected directly into heart muscle to promote angiogenesis therein.

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26. The method of claim 13, wherein the methods enhances collateral blood vessel formation.

27. The method of claim 13, wherein the method promotes development of newly implanted myocardial cells.

28. The method of claim 13, wherein the method promotes electrical conductivity of the heart of a patient with cardiac electrical pathway impairment.

29. The method of claim 13, wherein the method enhances myocardial function in a patient with impaired myocardial function.

30. The method of claim 13, wherein the method treats a left or right ventricular condition causing impaired heart function in the heart of the patient.

31. A composition comprising early attaching cells derived from autologous bone marrow, which cells have been transfected with a vector comprising a polynucleotide that encodes one or more agents selected from angiogenic cytokines growth factors, or angiogenesis-promoting factors.

32. The composition of claim 30, wherein the one or more agents are selected from HIF-1, EPAS1, MCP-1, and CM-CSF.

33. The composition of claim31, wherein the polynucleotide further comprises a Transcription regulatory region operatively associated with the polynucleotide.

34. The composition of claim31, wherein the transfected cells have been stimulated by exposure to hypoxia.

35. The composition of claim31, further comprising heparin or anotheranticoagulent.

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36. The composition of claim31, wherein the vector is an adenoviral vector.

37. A method for enhancing collateral blood vessel formation in a patient in need thereof, said method comprising: directly administering to a desired site in the patient an effective amount of bone marrow cells obtained from autologous bone marrow, and directly administering to the desired site in the patient an effective amount of RGTA, thereby enhancing collateral blood vessel formation at the site in the patient.

38. The method of claim 37, wherein the RGTA isRGTA11.

39 The method of claim38, wherein the bone marrow cells and theRGTA11 are injected into the site simultaneously.

40. The method of claim 37, wherein the desired site is a location of muscle ischemia.

41. The method of claim 41, wherein the muscle ischemia is in the heart of the subject.